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COCCAL FORMS OF THE DIPHTHERIA BACILLUS.

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For several years diphtheria toxin, prepared from the "Park and Williams No. 8" strain of *C. diphtheriæ*, has been used for the immunization of horses at these laboratories. From time to time smears of broth cultures of this organism used for toxin production have contained coccoid forms resembling micrococci, although irregularity in outline or staining, or the presence of intermediate forms between bacilli and cocci, has usually suggested a bacillary origin. These cultures of doubtful purity, when plated on Loeffler's blood-serum, have shown only characteristic dome-shaped colonies consisting entirely of bacilli.

Coccoid forms of diphtheroids have been described by Mellon (1917). Heinemann (1917) noticed the replacement of typical bacillary forms by coccus forms in certain of his cultures on beef, veal or horse-meat broth. His coccus-like growths produced similar toxin to his bacillary forms but of relatively low potency. On Loefflers's blood-serum and veal glucose agar

plates bacilli were present; on veal glucose agar slants they were invariably replaced by a certain number of coccal forms.

Crowell (1926) has studied the variants from a single virulent diphtheria bacillus, and has described coccoid forms, both virulent and avirulent, on Loefflers' blood-serum slants amongst the descendants of the original organism. He concludes that a virulent organism under favourable conditions always throws virulent offspring, but under unfavourable conditions virulent and avirulent, and that "morphological types of the diphtheria bacillus have no hereditary significance, and have no relation to virulence."

Yarisawa (1926) has described coccoid formation in 14 % of his collection of strains isolated from diphtheria patients. This was manifested only on certain media, especially in fresh blood-agar cultures transplanted from blood-serum media. When a suspension of coccal forms was injected into experimental animals there was definite evidence of toxicity, and bacillary forms were recovered post-mortem.

Yarisawa believes that insufficient heating of serum media is an important factor in inducing the coccal change, and was able to produce coccal forms of some of his strains by using insufficiently-heated Loeffler's medium. In ordinary bouillon he observed bacillary forms only.

The broth used for toxin-production at these laboratories has been Douglas's tryptic digest, modified by Hartley, and subsequently by Watson and Wallace. Digestion has usually been allowed to take place at 37°–40° C. for 3½ hours, but recently a three days' digest of horse-muscle at room temperature has been tried with favourable results. In the latter medium coccal forms are rather more frequent, but even then are rarely seen. A culture was obtained, however, some months ago consisting almost entirely of them. Through the kindness of my colleagues, Dr. A. F. Watson and Miss E. D. F. Langstaff, I have been enabled to study this culture in detail.

The culture consisted of a five days' growth of the organisms at 34° C. on a three days' tryptic digest of horse-muscle at room temperature. The medium had been sterilized by filtering through a Seitz press and autoclaving for twenty minutes at a pressure of 11 lb.

CHANGES OF MORPHOLOGY ON DIFFERENT MEDIA.

The original broth culture consisted for the most part of minute bodies arranged in clumps (Fig. 1). Many also were in diplococcal or streptococcal formation as if arranged along the bodies of bacilli, although the intermediate protoplasm was not seen. They only feebly resisted decolorization by Gram's method, and did not show metachromatic staining with Neisser's stain. A few slender and indistinct rod-shaped organisms were also present.

Subcultures were made from the original broth culture on to tubes of various media, from which plates were also prepared at frequent intervals to establish the continued purity of the strain. The main implantations were from (1) Loeffler's medium to Loeffler's medium, and (2) agar, prepared with trypsin digest broth as basis, to agar, with subcultures from time to time on to trypsin digest broth, serum digest broth, glucose digest broth, steamed Witte peptone broth, Witte peptone agar, and blood-agar. The Loeffler's medium was prepared by the addition of one part of sterile glucose digest

broth to three parts of normal horse-serum, the mixture being inspissated at 80° C. for one hour on two consecutive days.

On Loeffler slants and plates the sudden disappearance of the coccoid forms was most striking. The first generation consisted of Neisser-negative bacilli with some degree of beading; the second and subsequent generations showed typical Neisser-positive bacilli (Fig. 2).

On nutrient agar, prepared with tryptic digest broth as basis, coccal forms persisted for several generations (Figs. 3 and 4). They became steadily less numerous, until by the sixth successive subculture they had disappeared. None of the bacillary forms on "digest agar" had metachromatic bodies which could be stained by Neisser's method. It was found, however, that by subculturing from agar on to Loeffler's medium typical Neisser-positive organisms appeared within 24 hours. Subcultures of bacillary forms into digest broth led to the reappearance of the coccoid formation in the first generation (Fig. 5), the first coccoid forms being seen about half-an-hour after inoculation.

In the first transplant from the original broth on to agar, prepared with Witte peptone broth as basis, beaded bacilli with some degree of Neisser-positive staining displaced all the coccal forms (Fig. 6). In Witte peptone broth alone, however, two successive subcultures from the original broth contained numerous coccal forms. They also persisted in Hiss serum water, Witte peptone water, serum broth, glucose broth and blood-agar, suggesting that once an organism has assumed the coccal form, it tends to retain that formation unless it is placed on an eminently favourable medium such as Loeffler's blood-serum.

The colonies on plates of Loeffler's blood-serum were always bacillary. On plates of other media, however, colonies have been observed consisting of mixtures of coccal and bacillary forms. One batch of "digest agar" plates was of special interest. Two types of colonies appeared, one of which was much larger than the other; microscopically, one of these types was entirely bacillary, the other, the smaller variety, a mixture of coccal and bacillary forms.

A few freshly isolated strains of virulent and avirulent diphtheria bacilli and Hofmann's bacillus were subcultured on to the original broth (3 days' digest at room temperature), and assumed coccoid formation within 24 hours. Like the "Park and Williams No. 8" strain, they reverted almost at once to their typical morphology on a further transplantation to Loeffler's medium.

An attempt was made to induce coccoid formation direct from Loeffler slants by the use of media other than trypsin digest broth. As recommended by Yarisawa (1926), Loeffler's medium, which had been over-heated or insufficiently heated, and fresh blood-media, especially guinea-pig blood-agar, were inoculated with certain virulent and avirulent strains. It was found that by alterations in the medium, variations in morphology could readily be induced, but in no instance was there the extreme morphological change observed in the original batch of digest broth.

It should be stressed at this point that on Loeffler's medium, in our experience, even when a few cocco-bacillary or coccoid forms were present, the

remainder of the organisms were so nearly typical that the identification of the organism as *C. diphtheriæ* was never in doubt.

Miss Langstaff has occasionally observed marked coccal formation in one bottle of a toxin batch, inoculated at the same time and under the same conditions as many others in which only bacillary forms were present. In an attempt to explain this variation, experiments were carried out with negative results with dirty bottles and bottles containing fat or soap; nor did an increase in the phosphate or chloride content of the broth nor the addition of fruit or vegetable juice induce the coccal change.

VIRULENCE AND TOXIN PRODUCTION OF COCCAL FORMS.

Coccal forms of "Park and Williams No. 8" and two other strains which had been virulent when first isolated in the bacillary form were re-tested for virulence. Washed suspensions of the growths in digest broth were found to be fully virulent by the subcutaneous and intracutaneous methods of testing. In the subcutaneous test, organisms recovered from the site of injection were mainly bacillary in shape, indicating a tendency to reversion in the living body.

Forty-eight colonies from Loeffler plates, which had been inoculated from broth-cultures of three strains showing coccal forms, were also tested for virulence by the intracutaneous method. All were fully virulent, there being no evidence of any variation from colony to colony. These observations were the outcome of Crowell's hypothesis that a virulent organism under favourable conditions always throws virulent offspring, and under unfavourable conditions virulent and avirulent; once avirulent they do not again become virulent. If coccal formation is a response to an unfavourable environment, on this hypothesis one would have expected some of the colonies on the Loeffler plates to be avirulent, or at all events of intermediate virulence. No variation in virulence was, however, observed.

On several occasions good toxin has been obtained from cultures containing coccal forms. Whenever a bottle containing coccal forms has not produced as highly toxic a filtrate as other bottles of the same batch containing bacillary forms only, the growth in that bottle has been less vigorous—that is, fewer organisms were present. We do not know, therefore, if coccal forms individually are as good toxin-producers as bacillary forms.

SUMMARY.

(1) Coccal forms have appeared from time to time in tryptic digest-broth cultures of *C. diphtheriæ*.

(2) A culture of the "Park and Williams No. 8" strain on a 3 days' tryptic digest of horse-muscle at room temperature consisted almost entirely of coccal forms. This was a unique instance of extreme coccoid formation amongst several thousand broth-cultures examined. A large number of coccal forms were also produced when recently isolated virulent and avirulent strains were sown in this broth.

(3) The coccal forms disappeared on the first subculture on Loeffler's medium and on agar prepared with Witte peptone broth as basis. They were still

present in the fifth generation on agar prepared with digest-broth, but had disappeared in the sixth.

(4) Typical bacillary forms were most readily induced on Loeffler. Although coccal forms have been observed on this medium when it has been insufficiently heated, the remainder of the organisms have been so nearly typical that the identification of the cultures as *C. diphtheriæ* was never difficult.

(5) Coccal forms are virulent and toxigenic. There is no evidence that they are indicative of racial degeneration of the organisms.

I wish to thank Dr. A. F. Watson and Miss E. D. F. Langstaff for the original culture, estimations of toxicity of filtrates, and much helpful criticism. I am indebted to Dr. A. C. Stevenson of the Wellcome Bureau of Scientific Research for the micro-photographs.

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DESCRIPTION OF PLATE.

Fig. 1.—Tryptic digest broth culture. Gram stain.
Fig. 2.—4th subculture on Loeffler's medium. Methylene blue stain.
Fig. 3.—1st subculture on agar (tryptic digest broth basis). Neisser's stain.
Fig. 4.—3rd subculture as in Fig. 3.
Fig. 5.—Subculture from Loeffler's medium to broth as in Fig. 1.
Fig. 6.—1st subculture on agar (Witte peptone broth basis). Gram stain.

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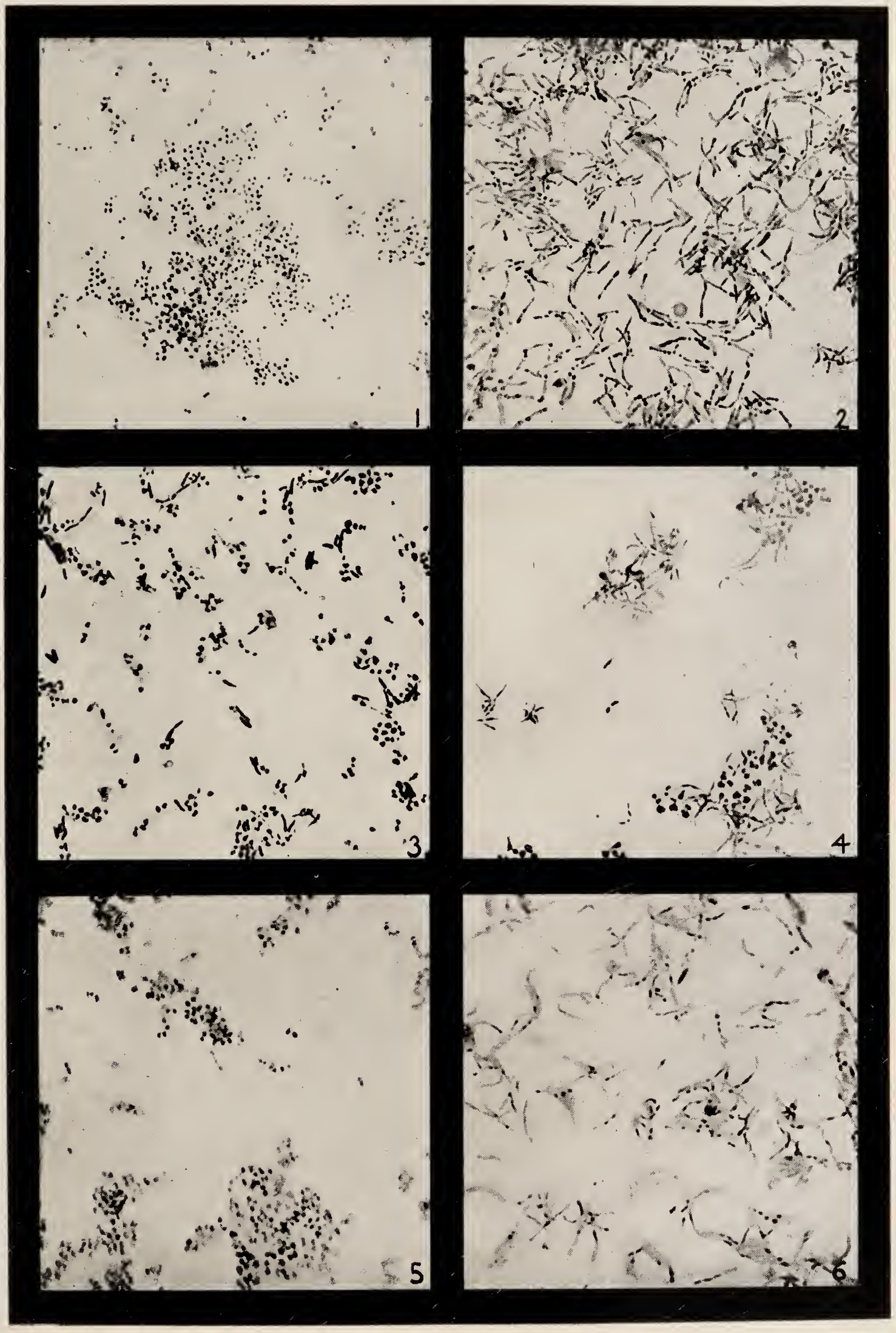

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